Intestinal and hepatic expression of BNIP3 in necrotizing enterocolitis: regulation by nitric oxide and peroxynitrite

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Necrotizing enterocolitis (NEC) is the most frequent and lethal disease affecting the gastrointestinal system of premature infants (16). The overall mortality rate for patients with NEC ranges between 10 and 70% (25) and approaches 100% for patients with the most severe form of the disease, which is characterized by involvement of the entire bowel (pan-necrosis; see Ref. 42). Although the exact etiology of NEC remains undefined, numerous risk factors, including prematurity (53), hypoxia (29), formula feeding (18), bacterial infection (43), and intestinal ischemia (1), have been implicated in its pathogenesis. These factors, together with the increasing number of associated inflammatory mediators (7, 21, 38), make NEC one of the most complex diseases in the neonatal population.

Among the proinflammatory mediators associated with the development of NEC is nitric oxide (NO), produced by the inducible NO synthase (iNOS). The end products of NO metabolism have been measured in newborn infants and in adult patients with clinical sepsis (40, 45). In an animal model of NEC induced by formula feeding and a brief period of hypoxia (3), we have recently found increased formation of endogenous nitroso species (mostly S-nitrosothiols, nitroamines, and nitrosylhemes), indicative of nitrosative stress (59). We have previously shown that NO modulates intestinal changes in septic shock (17, 46). Furthermore, iNOS is up-regulated in the intestine of infants with acute NEC, and this expression is downregulated by the time of stoma closure when the infant has recovered from the acute inflammatory insult (17).

Hypoxia leads to the production of oxygen radicals (5) and elevated iNOS expression (58). The reaction product of NO with superoxide (54), or through the action of neutrophil myeloperoxidase (23, 44) is peroxynitrite (ONOO•−; see Refs. 4 and 37). NEC is preceded by increased epithelial apoptosis, which results in loss of gut barrier function and thus predisposes the host to bacterial invasion and subsequent bowel necrosis (27). ONOO•− produced from iNOS-derived NO has been implicated in this process because it causes apoptotic cell death of enterocytes subjected to the inflammatory stimuli that characterize NEC (17).

An elevated level of NO has been shown to induce apoptosis in some cell types (e.g., macrophages) but to suppress apoptosis in others (hepatocytes; see Refs. 50 and 51). The upregulation of iNOS in the liver serves an anti-apoptotic function during experimental endotoxemia, a phenomenon that is mediated by S-nitrosation of caspases, production of cGMP, and modulation of apoptosis-related genes in endotoxemia. In states of redox stress such as hypoxia/ischemia reperfusion injury (50, 51), however, iNOS is hepatotoxic. We have shown that NO suppresses expression of the Bcl-2 binding protein BNIP3, a pro-apoptotic member of the Bcl-2 family of apoptotic factors, in hepatocytes (57). Both apoptotic and necrotic cell death mechanisms have been attributed to BNIP3 (9, 49), but the exact pathway(s) by which BNIP3 expression induces cell damage remains unknown.

The foregoing studies suggest a link between hypoxia and the intestinal production of nitroso species with the histopathological changes seen in NEC. Furthermore, these events may be modulated, in part, by BNIP3. In the present study, we pursued the hypothesis that BNIP3 is elevated in the intestine and liver of neonatal rats subjected to hypoxia and formula feeding. We show that both BNIP3 mRNA and protein are

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upregulated in the ileum and liver of formula-fed newborn rats exposed to hypoxia but not in breast-fed controls. In rats, this upregulation is reversed by an iNOS-specific inhibitor. We also show that BNIP3 is expressed in the human intestine and upregulated in intestinal tissue from human infants with acute NEC. Finally, ONOO− causes a time- and dose-dependent increase in BNIP3 protein in human enterocytes. We propose that hypoxia and subsequent formation of reactive oxygen and nitrogen species promote gut barrier failure in NEC by inducing enterocyte apoptosis, in part caused by elevated expression of BNIP3.

MATERIALS AND METHODS

Animal model of NEC. All experiments were carried out following an animal protocol approved by the Animal Research and Care Committee of the Children’s Hospital of Pittsburgh. Pregnant timedated Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were induced at term using a subcutaneous injection (1–2 U/animal) of Pitocin (Monarch Pharmaceuticals, Bristol, TN). Immediately after birth, the neonates were weighed and randomized into one of the different treatment groups. Group 1 consisted of neonatal rats left with their mothers, and thus they were breast-fed (BF). Group 2 consisted of neonates separated from their mothers, housed in a temperature- and humidity-controlled incubator (Ohio Medical Products, Madison, WI), gavaged with a special rodent formula (0.2 ml, see below) two times per day, and subjected to 10 min of hypoxia (5% O2–95% N2; Prax Air, Pittsburgh, PA) three times daily in a modular chamber (Billups-Rothenberg, Del Mar, CA) as follows: pups were fed in the morning posthypoxia, exposed to a second hypoxic insult after 4 h, and then subjected to the final hypoxic insult followed by the final feed. In some cases, the rats were treated daily after the first feeding with the iNOS inhibitor l-Arb-1-iminoethyllysine (l-NIL, 1 mg/kg ip; Alexis Biochemicals, San Diego, CA). The formula composition consists of 15 g Similac 60/40 (Ross Pediatrics, Columbus, OH) in 75 ml Esbilac canine milk replacer (Pet-Ag, Hampshire, IL) as described by Barlow et al. (3) and was designed to approximate the protein and caloric content of rat breast milk. The rats were killed on different days as indicated, and the intestinal samples (segments of terminal ileum) were harvested for morphological studies and Northern or Western blotting as described (38).

Collection of human intestinal specimens. The Human Rights Committee of Children’s Hospital of Pittsburgh approved collection of operative specimens for experimental purposes (Protocol No. 02–208). We compared six newborn patients undergoing bowel resection for NEC with six control neonates undergoing intestinal resection for inflammatory conditions other than NEC. Control patients consisted of patients undergoing intestinal resection for the following reasons: intestinal atresia, imperforate anus, intussusception, or meconium ileus, as we reported previously (17). In some instances, we examined intestinal tissue from infants who had recovered from intestinal perforation secondary to NEC who were now undergoing intestinal stoma closure. As such, they provided an internal control for these patients, since their tissues were examined at the time of initial presentation (acute NEC) and at the time of stoma closure when they had recovered from the disease. Diagnosis of NEC was confirmed historically by the hospital pathologist. At laparotomy, representative ileal segments were snap-frozen in liquid nitrogen and stored at −80°C until further analysis. Protein isolation and Western blotting analysis was performed as described below.

Morphological evaluation of intestinal samples. Rats were killed on different days, as indicated. The intestines were inspected for gross morphological changes, including pneumatosis intestinalis; the last 2 cm of terminal ileum were harvested for morphological studies. Hematoxylin and eosin slides were prepared as per standard protocol (13) and examined by light microscopy. The presence of morphological changes suggestive of human NEC in the neonatal rat intestinal epithelium, including separation of the villous core, submucosal edema, and epithelial sloughing, was determined and graded by a pathologist (Dr. R. Jaffe) from Children’s Hospital of Pittsburgh blinded to the experimental groups, as previously described (38).

Protein isolation and Western blot analysis. Newborn rats were killed as indicated, and segments of the terminal ileum were isolated. The mucosa was scraped gently from each segment and immediately placed in cold lysis buffer containing 62.5 mM Tris (pH 6.6), 10% glycerol, 1% SDS, and protease inhibitors (10 μg/ml leupeptin, 5 μg/ml pepstatin, 2 μg/ml aprotonin, and 0.5 mM phenylmethylsulfonyl fluoride, all from Sigma, St. Louis, MO). The samples were then homogenized and boiled for 1 min followed by centrifugation at 10,000 g for 30 min to remove cellular debris. Protein concentration in the supernatant was determined using the bicinchoninic acid Protein Assay kit from Sigma, with BSA as standard. Protein samples (equivalent to 50 μg) were resolved on 12% SDS-polyacrylamide gels using a Bio-Rad mini-gel system (Hercules, CA) and then electroblotted on polyvinylidene difluoride membranes (Millipore, Bedford, MA). After being blocked for 1 h with milk (5% in PBS with 0.1% Tween 20) at room temperature, the membranes were probed for 1 h at room temperature with the primary antibody (polyclonal rabbit anti-human BNIP3 from BD Pharmingen, San Diego, CA) dissolved in 1% milk PBS-Tween at 1:2,000 dilution. The membranes were then thoroughly washed and incubated with the secondary antibody (horse-radish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG; Pierce, Rockford, IL) at 1:15,000 dilution (in PBS-Tween with 1% milk) for 1 h before detection. Protein bands were visualized using a SuperSignal chemiluminescence substrate (Pierce) according to the manufacturer’s instructions. For normalization, the membranes were stripped and reprobed with an anti-β-actin antibody from Sigma.

RNA isolation and Northern blot analysis. Total RNA was isolated from the ileum or ileal mucosal scrapings, colon, and liver samples using the RNAlater reagent (Ambion, Austin, TX) as per the manufacturer’s protocol. Northern blotting and analysis of BNIP3 mRNA levels in rat samples was carried out using a probe obtained as described previously (57). The relative amount of mRNA is presented as the ratio of BNIP3 mRNA to 18S RNA.

Cell culture. The human fetal nonmalignant primary intestinal cell line (H4 cells) was used to represent immature fetal enterocytes and was generously provided by Dr. W. Allan Walker (Massachusetts General Hospital-East, Charlestown, MA). The H4 cells (from passages <30) were grown in DMEM (GIBCO, Grand Island, NY) supplemented with 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES buffer, 0.1 mM nonessential amino acid, 1 mM sodium pyruvate, and 0.2 U/ml insulin in a humid atmosphere of 95% O2–5% CO2 at 37°C. ONOO− (Alexis Biochemicals) was added to the culture plates at different concentrations, and, for experiments under hypoxic conditions, culture plates were placed in a hypoxic chamber (1% O2) for the times indicated.

Viability assay. Cell viability was determined by the crystal violet method, as described previously (34). Briefly, cells were stained with 0.5% crystal violet in 30% ethanol/3% formaldehyde for 10 min at room temperature. Plates were washed six times with tap water. After being dried, cells were lysed with 1% SDS solution, and dye uptake was measured at 550 nm using a 96-well microplate reader. Cell viability was calculated from relative dye intensity and presented as percentages relative to control samples.

Statistical analysis. Results are expressed as means ± SE or SD as indicated. Differences among groups were analyzed by the Student’s t-test or one-way ANOVA followed by Tukey’s Test or Fisher’s Least Significance Difference Test where appropriate (SigmaStat 2.03; SPSS, Chicago, IL). Statistical significance was determined at the 95% confidence level (P < 0.05) in all cases.
RESULTS

Formula feeding plus hypoxia upregulate BNIP3 mRNA expression in an animal model of NEC. Based on our observation that moderate to severe epithelial damage occurs in the intestine of formula-fed rats exposed to hypoxia (FFH) by day 4 after birth, we examined the expression of BNIP3 in the intestine of FFH and BF newborn rats (control). Mucosal scrapings of the terminal ileum and segments of the colon and liver from each rat were harvested on day 4 and processed for RNA isolation followed by Northern blotting analysis. BNIP3 mRNA was detected as a 1.7-kb band and was significantly upregulated in the ileum (Fig. 1A), colon (Fig. 1B), and liver (Fig. 1C) of FFH animals compared with BF controls ($P < 0.001$ by Student’s $t$-test). Similar upregulation of BNIP3 mRNA was found in the whole ileum of FFH compared with BF neonatal rats as determined by real-time PCR (data not shown).

Formula feeding plus hypoxia upregulated ileal BNIP3 protein expression in experimental NEC in an NO-dependent manner. Because one of the hallmarks of NEC is extensive epithelial damage involving the distal small bowel, we examined the expression of BNIP3 protein in the ileal mucosal scrapings of BF and FFH animals. As shown in Fig. 2A, BNIP3 protein is constitutively expressed at very low levels in the ileum of BF animals by the first day of life and remains essentially the same until death. In contrast, the levels of BNIP3 protein are slightly increased in the FFH group and significantly different from BF at day 4 ($P < 0.05$). Consistent with the upregulation of BNIP3 mRNA (Fig. 1A), formula feeding and hypoxia also led to upregulation of BNIP3 protein in the ileal mucosal scrapings of newborn rats (Fig. 2B). The increased expression of BNIP3 protein correlated with upregulation of other pro-inflammatory mediators in the mucosal scrapings of the ileal samples and with the characteristic pathological changes of experimental NEC by day 4 (59, 61). Interestingly, treatment with L-NIL (Fig. 2B) significantly suppressed the upregulation of BNIP3 protein in the FFH animals ($P < 0.05$) and the incidence of intestinal inflammation [28.6 $\pm$ 11.3 vs. 61.3 $\pm$ 8% in FFH without L-NIL, $n = 3$ independent experiments, total no. of animals/group (FFH + L-NIL/FFH): 28/31]. This observation, which stands in stark contrast to the downregulatory effect shown in hepatocytes (57), suggests that iNOS-derived reactive nitrogen intermediates participate in the upregulation of the ileal BNIP3 protein expression observed in experimental NEC.

[@P16447791] Fig. 1. Formula feeding plus hypoxia upregulate Bcl-2/adenovirus EIB 19-kDa-interacting protein 3 (BNIP3) mRNA in an animal model of necrotizing enterocolitis (NEC). Newborn rats were randomized into the following 2 groups: breast-fed (BF) animals were left with their mothers, and formula-fed pups were exposed to hypoxia (FFH). Mucosal scrapings of the terminal ileum (A), segments of the colon (B), and liver samples (C) from each rat were harvested on day 4. The samples from the same experimental group were combined and processed for RNA isolation followed by Northern blotting and analysis for BNIP3 mRNA (20 $\mu$g total RNA/lane). The membranes were rehybridized with 18S oligonucleotide probe as a loading control. A representative blot ($n = 3$ animals/group) for each tissue and the densitometric quantitation of different independent experiments are shown. For the densitometric analysis of the blots, the ratio of signal intensity for BNIP3 to that of 18S is defined as 1 in BF control samples. Results are the means $\pm$ SE of 3 independent experiments (A; $n = 3$ animals/group), 2 independent experiments (B; $n = 5$ animals/group), 4 independent experiments (C; $n = 4$–8 animals/group). *$P < 0.001$ vs. BF (analyzed by Student’s $t$-test).

Hyoxia and ONOO$^{-}$ induce cell death and increase BNIP3 protein expression in human intestinal epithelial cells. Intestinal hypoxia is a major factor in the pathogenesis of NEC. Upregulation of iNOS and excess production of reactive nitrogen species have been associated with the development of this disease (17). To investigate the effects of hypoxia and reactive nitrogen species on the expression of BNIP3 in human intestinal epithelial cells, H4 cells were incubated in a hypoxic
Fig. 2. Formula feeding plus hypoxia upregulate ileal BNIP3 protein expression in an animal model of NEC. Effect of nitric oxide (NO). Newborn rats were randomized into BF and FFH groups. The terminal ileum of each rat was harvested on the day indicated, and the mucosal scrapings were processed for protein isolation followed by Western blotting and analysis for BNIP3 protein. A: time course effect of breast feeding and formula feeding plus hypoxia on BNIP3 protein expression. Top, blot with 3 animals per group per day. Relative protein expression values on the graph are band densities normalized to β-actin and represent means ± SE (*P < 0.05 vs. BF day 4, analyzed by one-way ANOVA followed by Tukey’s test). B: role of NO on BNIP3 protein expression in an animal model of NEC. Newborn rats were randomized into the following 3 groups: B, FFH, or with l-N^6-(1-iminoethyl)lysine treatment (FFH + l-NIL). The terminal ileum of each rat was harvested on day 4, and the mucosal scrapings were processed for protein isolation followed by Western blotting and analysis for BNIP3 protein. Top, representative blot of 6 independent experiments. Relative protein expression values on the graph are band densities normalized to β-actin and are means ± SE (5 independent experiments with n = 3–5 animals per group per experiment). P < 0.05 vs. BF (*) and FFH (**: analyzed by 1-way ANOVA followed by Tukey’s test).

DISCUSSION

NEC is a complex, multifactorial disease of newborns characterized by intestinal epithelial cell apoptosis and necrosis and impaired enterocyte migration and proliferation that ultimately result in sustained gut barrier failure. In this study, we investigated the expression and regulation of the cell death-related protein BNIP3 (19, 41, 49) in the intestine and liver of newborn rats in an experimental model of NEC, in human intestinal epithelial cells in vitro, and in the diseased intestine of human infants with acute NEC.
BNIP3 has been shown to be overexpressed in human tumors and human tumor cell lines (12, 30, 47, 48) and in primary neonatal rat cardiac myocytes exposed to hypoxia (19, 31). Furthermore, at normal oxygen concentrations, most tissues have undetectable levels of BNIP3 but activate transcription during hypoxia through a 5'H11032 promoter of a hypoxia-inducible factor 1 (HIF-1) binding site (see Ref. 52 for review). However, this is the first report of BNIP3 expression in the human intestine and in nontumor tissue in an experimental animal model.

BNIP3 is a membrane-associated protein localized to mitochondria and other cytoplasmic membrane structures and is widely expressed in a large number of mouse and human tissues (9). BNIP3 belongs to the BH3-containing Bcl-2 family proteins in which the BH3 domain plays an important role in eliciting apoptosis (55). However, a recent study shows that BNIP3 heterodimerizes with Bcl-2/Bcl-xl and induces cell death independent of a BH3 domain at both mitochondrial and nonmitochondrial sites (41). In addition to apoptosis, BNIP3 may also mediate a form of necrotic cell death after protein integration in the mitochondrial outer membrane and rapid mitochondrial permeability transition pore opening (49). Because NEC is characterized by an extensive hemorrhagic inflammatory necrosis of the distal small bowel and proximal colon (39) and is associated with epithelial apoptotic cell death (10, 27), it is not surprising that cell death-related genes such as BNIP3 are upregulated in the intestine of NEC patients. Interestingly, the expression of BNIP3 (both mRNA and protein) is induced and related to hypoxia-induced apoptosis in a number of human and animal cell lines, including: CHO-K1 (Chinese hamster ovary), CV-1 (monkey kidney), Rat-1 (rat fibroblast), PAM212 (human epithelial), Hep G2 (human hepatocellular carcinoma), and ECV-304 (human bladder carcinoma) cell lines (6). Moreover, BNIP3 and Nix (a BNIP3 homolog sharing both structural and functional similarity; see Ref. 8), are the only members of the Bcl-2 family of apoptotic factors induced in response to hypoxia (6). Whether the expression of BNIP3 is a relevant process in human NEC still needs to be investigated. We attempted to suppress the expression of BNIP3 in IEC-6 rat intestinal epithelial cells using a commercial small-interfering RNA (Silencer; Ambion) to determine the role of BNIP3 in enterocyte apoptosis, but were unsuccessful (data not shown).
Based on the findings that mucosal damage associated with enteral feeding and perinatal hypoxia are two major factors in the development of NEC, we used a reproducible experimental model in newborn rats developed in our laboratory (38) to study the expression of BNIP3. It has been previously shown that, in the liver, BNIP3 mRNA is expressed as a major transcript of 2.5 kb and as a minor transcript of 1.7 kb (9). Our results show that BNIP3 mRNA is expressed as a 1.7-kb transcript in the intestine of newborn rats and is increased in the FFH group compared with control animals on day 4. This finding correlates with the morphological changes characteristic of intestinal inflammation seen in ileal segments from FFH rats compared with ileal segments from BF controls. Consistent with the finding that the major intestinal damage in NEC occurs in the terminal ileum, we found a fourfold increase in BNIP3 mRNA in the ileal mucosal scrapings from FFH animals compared with only a twofold increase in colon samples from the same animals. It should be noted that, during the isolation of total RNA from ileal mucosal scrapings, the detectable yield was too low to perform individual analysis for each animal; therefore, the samples from the same experimental group had to be pooled for Northern analysis. Consequently, it is possible that the actual relative levels of BNIP3 mRNA in FFH animals may be higher than the values reported here.

In addition to intestinal epithelial injury, liver failure or dysfunction is also seen in severe NEC. Indeed, a role for hepatic inflammatory mediators contributing to intestinal damage in NEC has recently been postulated (20). The exact role of the liver in the pathogenesis of NEC, however, remains to be elucidated. Based on our demonstration that BNIP3 is expressed in liver hepatocytes isolated from adult rats subjected to pro-apoptotic stimuli (57), we hypothesized that BNIP3 is also expressed in the liver of newborn rats and that this expression is differentially regulated in FFH vs. BF animals. Our data show that formula feeding plus hypoxia upregulate BNIP3 mRNA expression in the liver of FFH animals compared with BF controls. The degree of increase was similar to that seen in the ileum, suggesting that BNIP3 is involved in the liver damage associated with NEC. Interestingly, our previous studies indicated that NO or its reactive metabolites suppress the expression of BNIP3 in hepatocytes in vitro (57), in contrast to our results in vivo in the liver of FFH rats. We speculate that the differential effects of NO in the liver may be context-dependent. paradoxically, NO is either pro- or anti-apoptotic, depending on cell type, dose, and milieu. For example, low levels of NO tend to protect various cell types from apoptotic death, whereas high levels are generally toxic (14). In vivo, NO can protect the liver in endotoxemia (22), yet exposure to NO leads to liver injury during ischemia/reperfusion (33). NO derived from iNOS is also hepatotoxic in whole animal models of ischemia/reperfusion such as hemorrhagic

Fig. 4. Peroxynitrite (ONOO−) alone or in combination with hypoxia causes cell death and upregulates BNIP3 protein expression in human intestinal epithelial cells. Primary human fetal intestinal epithelial cells (H4 cell line) were treated with ONOO− (1–50 μM) for 4 or 24 h, and the cells were harvested for protein isolation and Western blotting analysis. For hypoxia experiments, the culture plates were placed in a hypoxic chamber (1% O2) for the times indicated. Viability experiments were performed in parallel using the crystal violet method. A: Western blot showing the expression of the BNIP3 60-kDa band (duplicate samples/group). Results in bars represent means ± SD. *P < 0.05 vs. control and 1–25 μM ONOO− (analyzed by 1-way ANOVA followed by Tukey’s test) B: viability of H4 cells exposed to ONOO− (1–50 μM) for 24 h. Results in bars represent means ± SE of 5 independent experiments. *P < 0.01 and **P < 0.001 vs. control (analyzed by 1-way ANOVA followed by Tukey’s test). C: Western blot showing the effect of the combined treatment on BNIP3 protein expression. Results represent the band density expressed as degree of increased BNIP3 expression vs. hypoxia alone at the respective time point from 2 independent experiments. *P < 0.01 vs. hypoxia without ONOO−, **P < 0.05 vs. 25 μM ONOO−, ***P < 0.05 vs. 5 μM ONOO− (analyzed by 1-way ANOVA followed by Fisher’s LSD Test).
Fig. 5. BNIP3 protein expression in human NEC. Representative ileal segments from 6 newborn patients undergoing bowel resection for NEC (4–6 and 10–12) and ileal specimens from 6 neonates undergoing intestinal resection for inflammatory conditions other than NEC (control, 1–3, and 7–9) were analyzed for the presence of BNIP3 in 2 independent experiments. The frozen intestinal samples were processed, and Western blotting and analysis using a rabbit polyclonal antibody against human BNIP3 was performed as described in MATERIALS AND METHODS with 50 μg protein/lane. Results in bars represent means ± SE (n = 6 patients/group). *P < 0.05 vs. control (analyzed by Student’s t-test).
and thereby may provide a new therapeutic target for this disease.

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